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Abstract: BACKGROUND Sepsis is a pathological host response to infection leading to vascular barrier breakdown due to elevated levels of angiopoietin-2 (Angpt-2) and vascular endothelial growth factor-A (VEGF-A). Here, we tested a novel heterodimeric bispecific monoclonal IgG1-cross antibody of Angpt-2 and VEGF - termed "A2V." METHODS Cecal ligation and puncture was used to induce murine polymicrobial sepsis. Organs and blood were harvested for fluorescence immunohistochemistry and RT-PCR, and survival was recorded. In vitro endothelial cells were stimulated with plasma from septic shock patients costimulated with A2V or IgG antibody followed by immunocytochemistry and real-time transendothelial electrical resistance. RESULTS Septic mice treated with A2V had a reduced induction of the endothelial adhesion molecule ICAM-1, leading to a trend towards less transmigration of inflammatory cells (A2V: 42.2 ± 1.0 vs. IgG 48.5 ± 1.7 Gr-1+ cells/HPF, p = 0.08) and reduced tissue levels of inflammatory cytokines (e.g., IL-6 mRNA: A2V 9.4 ± 3.2 vs. IgG 83.9 ± 36.7-fold over control, p = 0.03). Endothelial permeability was improved in vivo and in vitro in stimulated endothelial cells with septic plasma. Survival was improved by 38% (p = 0.02). CONCLUSION Dual inhibition of Angpt-2 and VEGF-A improves murine sepsis morbidity and mortality, making it a potential therapeutic against vascular barrier breakdown.

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Keywords
Sepsis · Endothelium · Vascular leakage · Angiopoietin · Tie2 · Vascular endothelial growth factor

Abstract
Background: Sepsis is a pathological host response to infection leading to vascular barrier breakdown due to elevated levels of angiopoietin-2 (Angpt-2) and vascular endothelial growth factor-A (VEGF-A). Here, we tested a novel heterodimeric bispecific monoclonal IgG1-cross antibody of Angpt-2 and VEGF – termed "A2V." ***Methods:*** Cecal ligation and puncture was used to induce murine polymicrobial sepsis. Organs and blood were harvested for fluorescence immunohistochemistry and RT-PCR, and survival was recorded. In vitro endothelial cells were stimulated with plasma from septic shock patients costimulated with A2V or IgG antibody followed by immunocytochemistry and real-time transendothelial electrical resistance. ***Results:*** Septic mice treated with A2V had a reduced induction of the endothelial adhesion molecule ICAM-1, leading to a trend towards less transmigration of inflammatory cells (A2V: 42.2 ± 1.0 vs. IgG 48.5 ± 1.7 Gr-1$^+$ cells/HPF, $p = 0.08$) and reduced tissue levels of inflammatory cytokines (e.g., IL-6 mRNA: A2V 9.4 ± 3.2 vs. IgG 83.9 ± 36.7-fold over control, $p = 0.03$). Endothelial permeability was improved in vivo and in vitro in stimulated endothelial cells with septic plasma. Survival was improved by 38% ($p = 0.02$). ***Conclusion:*** Dual inhibition of Angpt-2 and VEGF-A improves murine sepsis morbidity and mortality, making it a potential therapeutic against vascular barrier breakdown.

Introduction

Sepsis is characterized as a life-threatening organ dysfunction caused by an overwhelming host response to an infection [1]. Sepsis is one of the most frequent causes of emergency admission and counts responsible for approximately 11% of all admissions to intensive care units in high-income countries [2]. Its incidence is rising [1, 3] and globally more than 5 million septic people die every

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single year [4]. The mortality rate has recently been found to be up to 59% in septic shock patients in Germany [3]. General therapeutic management is mostly based on the administration of anti-infectives and source control. Besides these direct treatments against the infection, only supportive strategies (fluid resuscitation, vasopressors, low tidal volume ventilation [5], etc.) have been established. So far, there is no specific agent to causally treat the pathological host response in sepsis. Despite intensive research, novel innovative therapies that worked in mice have largely failed to proof efficacy in clinical trials [6]. It has been assumed that the negative clinical trial results are due to poor animal models of sepsis [6] and the heterogeneity of the immune response in men – a fact that is further aggravated by the absence of monitoring tools of a single patients' immune status. What might help one patient at an early disease state might actually harm another. The need for a promising new therapeutic target that might be tailorable to individual needs of a given patient is more important than ever [7, 8].

The physiological vascular response to a local infection includes the controlled recruitment and transmigration of neutrophils both by activating the endothelium and then increasing its permeability. As part of the pathological (systemic) host response in sepsis, this highly regulated molecular mechanism regulating the physiological processes of local inflammatory responses occurs in a rather uncontrolled fashion, leading to global vascular leakage and massive infiltration of inflammatory cells. Together, this aggravates multi organ failure and contributes to death. This vascular response to infection is less heterogenous and appears to be continuously aggravating over time as the disease progresses. Therefore, the dysfunctional endothelium might represent a promising target for the development of novel therapeutic strategies against sepsis morbidity and mortality [9].

The vascular endothelial growth factor (VEGF)/VEGF receptor as well as the angiopoietin (Angpt)/Tie2 system are both ligand-tyrosine kinase receptor systems that are almost exclusively expressed in the vasculature and can modify endothelial inflammation, vascular hemostasis, vasomotor tone, and importantly the vascular barrier. In sepsis, it has been shown that circulating levels of VEGF-A [10, 11] and Angpt-2 [12, 13] are elevated (leading to activation of the VEGF receptor and deactivation of Tie2, respectively). These events contribute to endothelial barrier breakdown and tissue inflammation, leading to vascular leakage with hypovolemia, tissue edema, altered microcirculatory flow, and ultimately to multiple organ dysfunction.

Kienast et al. [14] used the complementary effects of VEGF-A and Angpt-2 on the vasculature to modulate neoangiogenesis in cancer. To target both ligands simultaneously, the authors developed a heterodimeric bispecific IgG1 antibody via the CrossMAb technology [15] termed Angpt2-VEGF CrossMAb (or short A2V). A2V combines bevacizumab on one arm and LC06 on the other arm. Over the last decade, we and others have shown that targeting Tie2 by modulating circulating ligands (e.g., genetic depletion, siRNA, synthetic activation, ABTAA) can improve survival in animal models of the disease by up to 50%. Interestingly, the effect of Angpt-2 inhibition by antibodies was only very mild or even absent [16]. Positive effects on survival and endothelial permeability in sepsis have also been reported for VEGF-A inhibition using bevacizumab [17].

Based on these facts and the theoretical benefits of an antibody strategy, we designed a preclinical proof-of-principle study to investigate the putative beneficial effect of dual inhibition of Angpt-2 and VEGF-A with a novel heterodimeric bispecific antibody (A2V).

Material and Methods

Antibodies and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

CrossMAb Antibody

The dual Angpt-2-VEGF-A (A2V) antibody (Roche No. R06872840-000-006) is based on a combination of LC06 (anti-murine/human Angpt-2) and B20-4.1 (anti-murine/human VEGF-A). Mouse isotype antibody of unknown specificity was used as control (Roche No. 0437-0004). Antibodies were dissolved in sodium chloride 0.9% and injected in mice intraperitoneally or were applied to human umbilical vein endothelial cell (HUVEC) culture. A2V CrossMAb and IgG control were kindly provided by pRED, F. Hoffmann LaRoche (Basel, Switzerland). A2V is generated as described elsewhere [18].

Animal Studies

All experiments were approved by the local committee for care and use of laboratory Animals (LAVES Lower Saxony, reference No. AZ/12-0681) and were performed according to international guidelines on animal experimentation. Male C57BL/6 mice (8–10 weeks old, weighing 20–25 g) were purchased from Charles River Laboratories (Sulzfeld, Germany) and Central Animal Facility of Hanover Medical School and maintained under standard conditions. One hour prior to polymicrobial sepsis induction by cecal ligation puncture (CLP), mice were treated with single intraperitoneal injection (30 mg/kg body weight) of IgG-control or A2V antibodies, respectively. Anesthesia was induced by 3.5% isoflurane and maintained with 1.5% isoflurane (Baxter, Unterschleißheim, Germany), shaving, and disinfection with 70% etha-

nol, then a median laparotomy was performed to expose the cecum. The cecum was then ligated and punctured once with a 20-G needle. For sham mice, this step was skipped. After closing the abdomen by two-layered suturing, all mice were resuscitated subcutaneously with 200 μL of 0.9% saline. To avoid hypothermia, mice were kept warm by an infrared lamp in the perioperative setting. Sixteen hours post CLP, mice were sacrificed to harvest organs or to perform functional analysis of permeability. Organs were either shock-frozen in liquid nitrogen for further molecular analysis or were collected in PBS for later paraffin embedding for histology.

Measurements of Serum Parameters

Serum analysis of lactate dehydrogenase, blood urea nitrogen, glutamate-pyruvate transaminase, and glutamic oxaloacetic transaminase were measured by an automated method using OLYMPUS AU 400 (Beckman Coulter, Krefeld, Germany).

RNA Purification and Quantitative PCR

The RNeasy Mini Kit (Qiagen, Hilden, Germany) and the RNeasy-Micro-Kit (Qiagen Technologies, Hilden, Germany) were used to extract total RNA from organ tissue or cultured cells, respectively, per manufacturers' instructions. Via the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Rotkreuz, Switzerland) added by FastStart Taq DNA Polymerase, dNTPack (Roche Diagnostics, Rotkreuz, Switzerland) 1 μg of total RNA was then reverse transcribed to cDNA followed by SYBR Green real-time quantitative PCR using LightCycler 480 II (Roche, Basel, Switzerland). For primers purchased from Qiagen, we used SYBR Premix Ex Taq II Takara (CLONTECH TAKARA) as DNA polymerase. For all other primers, we used TaqMan® Universal Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The following specific primers were used for quantification: murine β-actin (Fw: CCT GAG CGC AAG TAC TCT GTG T; Rev: CTG CTT GCT GAT CCA CAT CTG), murine Angpt-2 (Fw: GCT GAA GGA CTG GGA AGG C; Rev: GGA CTC TTC ACC AGC GAG GTA), murine intercellular adhesion molecule-1 (ICAM-1; detected transcript QT00155078; Qiagen, Hilden, Germany), murine tumor necrosis factor (TNF)-α (detected transcript QT00104006; Qiagen, Hilden, Germany), murine interleukin (IL)-6 (detected transcript: NM_031168; Qiagen, Hilden, Germany), murine vascular cell adhesion molecule-1 (VCAM-1; Fw: ACA TCC CTC CAC AAG GCT TCA AGA; Rev: TGG CAT TTC CTG AGA GAA GCT GGA), human β-actin (Fw: CTG GAA CGG TGA AGG TGA CA; Rev: AGT CCT CGG CCA CAT TGT G).

For each sample, triplicate RT-qPCR analyses were performed, and the mean of the obtained threshold cycle values (CT) was taken. Gene expression was standardized to the expression of the housekeeping gene (murine or human β-actin), yielding the ΔCT value.

Primary HUVEC Culture

Informed consent was obtained from all HUVEC donors and approved from the ethical committee of Hannover Medical School (No. 1303-2012). Cell isolation was conducted in accordance with institutional and governmental guidelines. Cells were isolated from human umbilical veins with the help of heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), phosphate-buffered saline (Thermo Fisher Scientific, Waltham, MA, USA), trypsin/ethylenediaminetetraacetic acid solution (Biochrom, Berlin, Germany), and collagenase (Biochrom, Berlin, Germany). Cells were used at passage P3–P5. Culturing was performed in endothelial cell growth medium containing 2% fetal bovine serum according to the manufacturer's instructions (Lonza, Basel, Switzerland). Unless otherwise stated, HUVECs were stimulated with 50 ng/mL recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) to induce a sepsis-like phenotype.

Fluorescent Immunohistochemistry in HUVECs

Coverslips were coated with collagen (Sigma-Aldrich, St. Louis, MO, USA), upon which HUVECs were grown to confluency. Cells were then preincubated with starvation medium (EBM2 + 0.5% FCS + gentamicin) for 3.5 h. Afterwards, either 4 μg/mL IgG control or A2V was added for 1 h followed by stimulation with 5% septic plasma for 30 min. At the end of the experiment, cells were fixed with 4% paraformaldehyde, blocked with 10% donkey serum (Jackson Immuno Research Inc., West Grove, PA, USA) and permeabilized with 0.1% Triton X-100 in PBS (Sigma-Aldrich, St. Louis, MO, USA). Coverslips were then incubated with primary antibody against VE-cadherin (CD144; BD Pharmingen, Heidelberg, Germany) for 1 h at room temperature. As a secondary antibody, anti-Actin Alexa Fluor 546 Phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) was used. 4′,6-diamidino-2-phenylindole (DAPI; SIGMA-Aldrich, St. Louis, MO, USA) for 10 s stained the nucleus. Finally, the coverslips were affixed with aqua-poly/mount (Polysciences Inc., Eppelheim, Germany). The images were taken with a Leica DMI 6000B microscope and were all obtained with the same gain, exposure, and offset conditions.

Fluorescent Immunohistochemistry

Paraffin-embedded sections (1.5 μm) from lung and kidneys were labelled with primary antibody against Gr-1 (AbD serotec, Puchheim, Germany). As secondary antibodies, we used goat anti-rat IgG-HRP (Santa Cruz Biotechnology, CA, USA). Cryosections (6 μm) were blocked with 10% donkey serum (Jackson Immuno Research Inc., West Grove, PA, USA) and stained with primary antibody against ICAM-1 (or M19; Santa Cruz Biotechnology). As secondary antibody, goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA, USA) was used. For global histomorphologic analysis of lungs, we used periodic acid-Schiff (PAS) staining.

Electric Cell-Substrate Impedance Sensing

Transendothelial electrical resistance (TEER) was measured using an electric cell-substrate impedance sensing (ECIS) system (Applied BioPhysics Inc., New York, NY, USA). Values were pooled at discrete time points and plotted versus time. Each conditions' end resistance was divided by its starting resistance to give the normalized TEER. Confluence was determined by the monolayer achieving manufacturer-recommended electric criteria.

Septic Shock Patients

To generate an in vitro assay to investigate the phenotype of the septic endothelium, we stimulated HUVECs by supplementing the media with 5% plasma from two different patients suffering from early-onset (<12 h) and very severe (norepinephrine dose >0.4 μg/kg/min) septic shock. The plasma was originally obtained in the context of another study (No. 2786-2015) [19]. Patients agreed with this additional experiment.

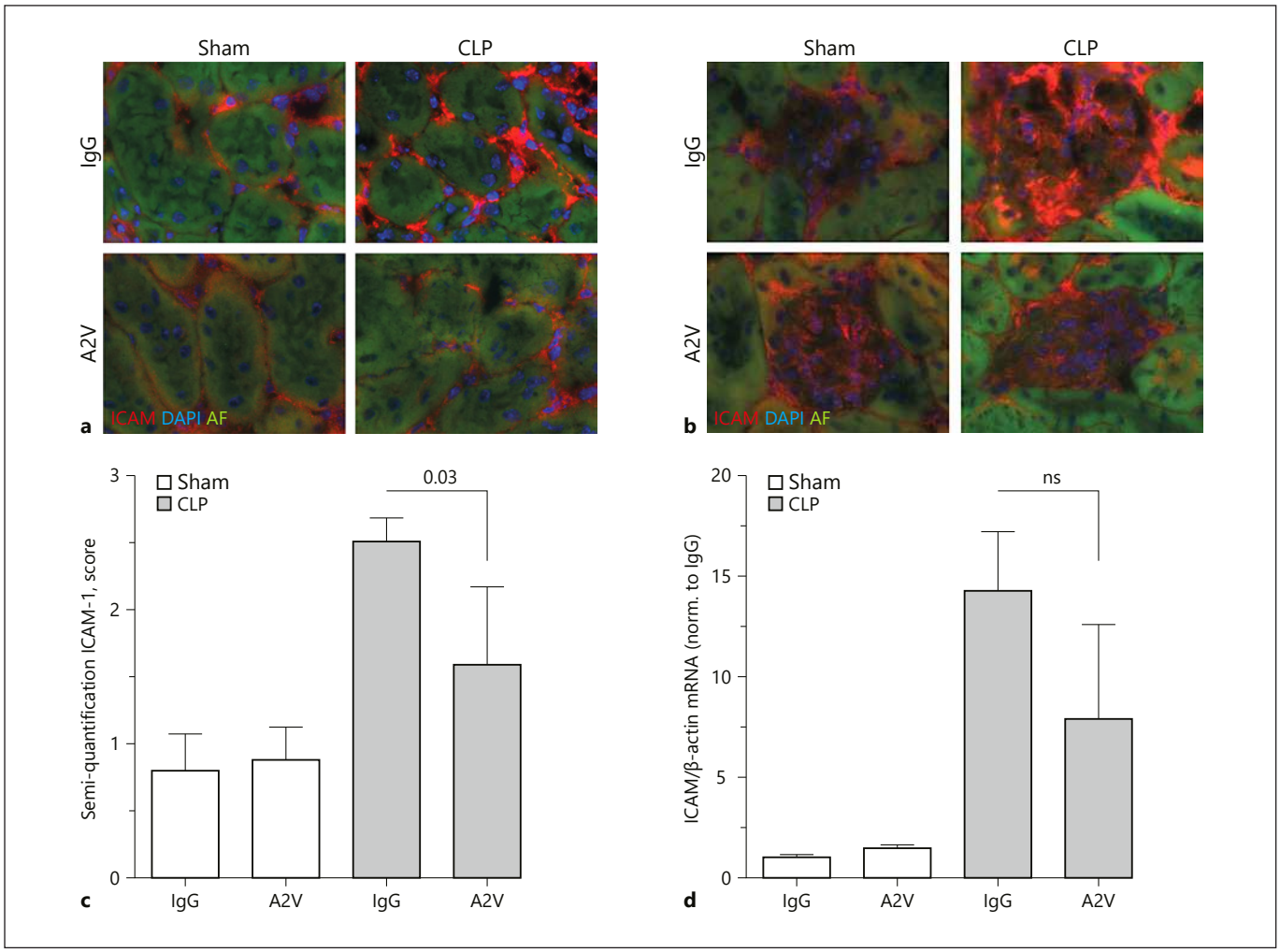

Fig. 1. A2V modulates endothelial adhesion molecule expression. Fluorescent immunohistochemistry for intercellular adhesion molecule (ICAM)-1 (red) was performed 16 h after cecal ligation puncture (CLP) or sham surgery in A2V (30 mg/kg) or IgG control antibody (IgG)-treated mice (nuclear staining with 4′,6-diamidino-2-phenylindole, blue; autofluorescence is shown in green). Representative results for each group (n = 6–8) are shown in peritubular (**a**) or glomerular regions (**b**), respectively. **c** Semi-quantification of whole kidney cross-sections was assessed by a single blinded operator ranging from 1 = mild, 2 = moderate to 3 = severe. **d** Real-time quantitative polymerase chain reaction in kidney was used to quantify ICAM-1 messenger RNA (mRNA) as in the above-described conditions (n = 8).

Statistical Analysis

For all analysis and graph creation, we used Graph Pad Prism 5 (La Jolla, CA, USA). Results are expressed as mean ± SD unless otherwise noted. Differences between groups were calculated with one-way ANOVA and non-parametric Mann-Whitney U test and considered significant with two-tailed p value <0.05. Survival data were analyzed through a log-rank test and visualized by Kaplan-Meier curves. Serum measurements were analyzed with unpaired t test with Welch's correction.

Results

A2V Modulates Endothelial Adhesion Molecule Expression

Given the importance of endothelial activation in the process of recruiting leukocytes to local sites of tissue inflammation, we first analyzed the expression of adhesion molecules in murine experimental sepsis. Fluorescent immunohistochemistry in renal tissue revealed a marked increase in peritubular and glomerular ICAM-1 expression in CLP sepsis (Fig. 1a, b, upper panels), which was

markedly reduced in the A2V-treated animals compared to IgG control (IgG)-treated ones (Fig. 1a, b, lower panels, and Fig. 1c). Additionally, RT-PCR revealed a trend towards reduced ICAM-1 (Fig. 1d) and VCAM-1 mRNA expression in whole kidney lysates.

A2V Reduces Tissue Infiltration of Inflammatory Cells and Cytokine Production

Next, we investigated if this reduction in adhesion molecule expression led to reduced transmigration of inflammatory neutrophils and less tissue cytokine production. Given that the highest number of transmigrated neutrophils can regularly be seen in lungs, we primarily investigated the pulmonary capillaries for Gr-1$^+$ cell infiltration by fluorescent immunohistochemistry. Upon experimental sepsis induction, Gr-1$^+$ cells were increasingly recognized in the alveolar capillary space (Fig. 2a, highlighted by white arrowheads). This influx of Gr-1$^+$ cells was noticeably reduced in A2V-treated animals (Fig. 2a, lower right image). Semi-quantification of Gr-1$^+$ neutrophil expression revealed a trend towards reduction upon A2V treatment (Fig. 2b). The same observation was true for local production of a key cytokine of sepsis, i.e., IL-6 investigated by RT-PCR on mRNA level in kidneys and lungs (Fig. 2c, d). Similar trends were seen for TNF-α.

A2V Affects Permeability in vivo and in vitro

To asses vascular permeability in vivo, we used classical histology with PAS staining to assess a phenomenon termed "peribronchial cuffing," which visualizes the presence of lung edema on the morphological level. Figure 3a shows representative slides obtained from septic and sham mice treated with A2V or IgG. The occurrence of cuffing is highlighted with red arrows around the arteriolae (as vasa vasorum of the adjacent bronchus – that also both share one adventitia). Semi-quantification revealed not only a significant increase of cuffing upon sepsis induction (sham: 0.6 ± 0.4 vs. CLP: 2.5 ± 0.5, $p < 0.0001$) but also a reduction in the A2V group compared to IgG (A2V: 1.4 ± 0.3 vs. IgG: 2.5 ± 0.5, $p = 0.002$) (Fig. 3b).

To directly test a potential anti-permeability effect of A2V, we used in vitro models with stimulated HUVECs and assessed morphological changes by fluorescent immunocytochemistry of a major adherens junction protein (i.e., VE-cadherin – in green) as well as the cytoskeletal architecture by F-actin (in red). To induce the phenotype of a septic endothelium with vascular leakiness, we challenged the HUVECs with plasma from 2 exemplary septic shock patients. Both upper panels of Figure 4a show the typical cellular correlate of barrier dysfunction (i.e., disruption of VE-cadherin and the formation of actin stress fibers together leading to gaps between adjacent cells). These morphological changes are protected when cells were co-incubated with A2V (lower 2 panels). Here, VE-cadherin is continuously expressed at cell-cell contacts and F-actin is configurated in a cortical manner, thereby generating a force that presses each cell towards another. To quantify these qualitative observations, we used real-time measurements of the endothelial integrity by TEER measurements using the ECIS device. Adding septic plasma to endothelial cells induced an acute drop in resistance (i.e., permeability) that could be prevented when the cells were co-incubated with A2V (Fig. 4b).

Effect of A2V on Organ Function and Survival

Sixteen hours after sepsis induction, we observed a slight increase in blood urea nitrogen as an indicator of a beginning acute kidney injury. Liver enzymes and lactate dehydrogenase as global markers for tissue injury were analogously elevated. All these surrogates of organ dysfunction showed trends towards improvement in the A2V-treated group (Table 1). Lastly, we analyzed if the earlier described effects on functionally relevant changes in experimental sepsis might indeed improve clinically relevant outcomes. The Kaplan-Meier graph in Figure 5 shows the survival of A2V-pretreated mice (–1 h) compared to the IgG control antibody (IgG) in a polymicrobial CLP sepsis model. Two different A2V doses (10 mg/kg [Fig. 5a] and 30 mg/kg body weight [Fig. 5b]) were assessed. The higher dose showed a survival benefit of 36.4% ($p = 0.02$) in a CLP model adjusted to a 90% mortality.

Discussion

Here, we tested for the first time a dual pharmacological blockade of Angpt-2 and VEGF-A in a polymicrobial murine model of experimental sepsis using a novel heterodimeric bispecific monoclonal antibody. Although we and others have proposed that Angpt-2 directly contributes to sepsis morbidity and mortality [12, 13], treatment strategies with monoclonal antibodies (as a delivery platform) against Angpt-2 have been mostly disappointing. Therapeutic VEGF blockade by antibodies has also shown mixed results [17, 20–22]. Here we found that dual blockade of both Angpt-2 and VEGF-A led to reduced endothelial inflammation and vascular permeability, leading to an improved survival by 36.4%.

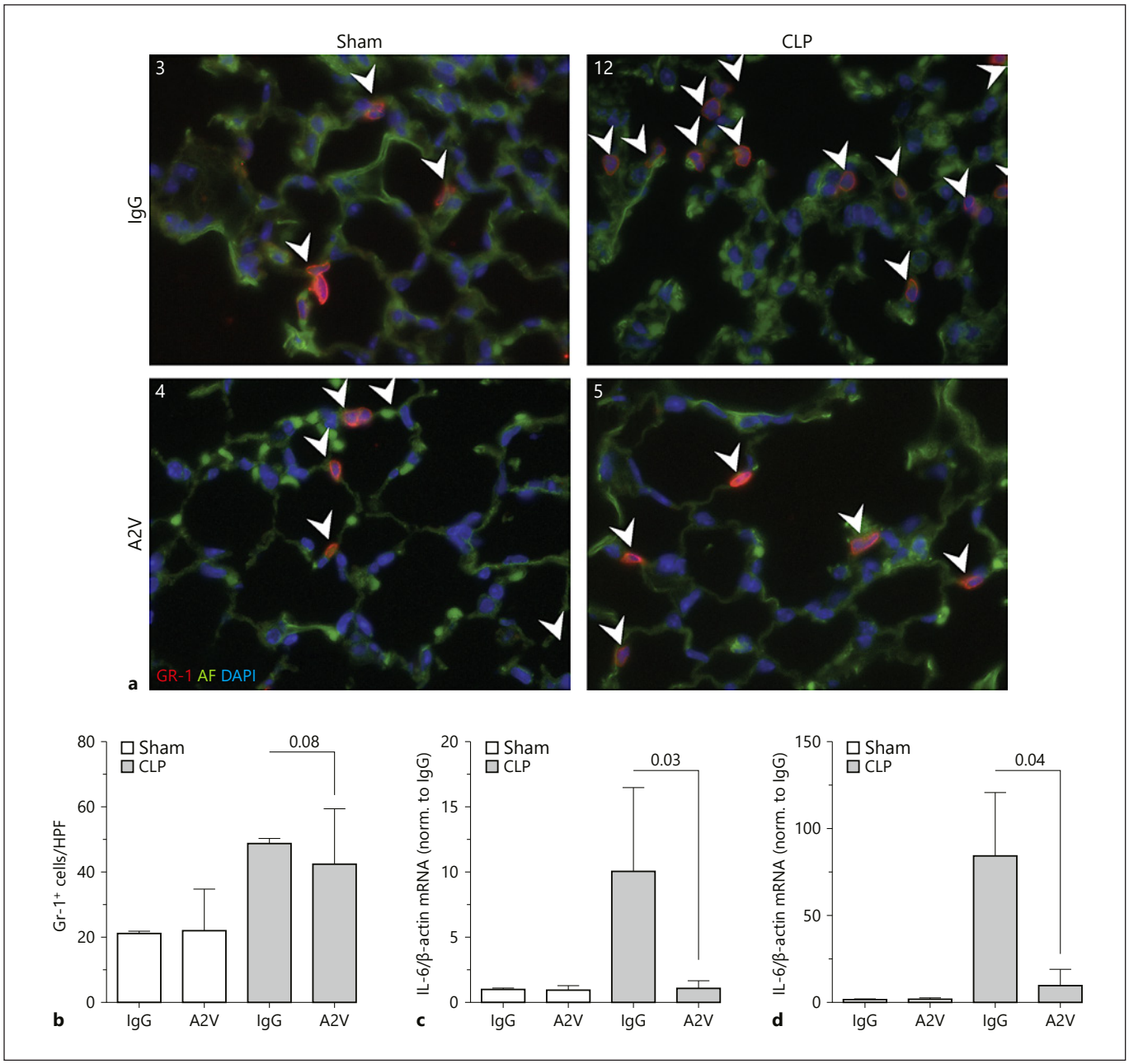

Fig. 2. A2V reduces tissue infiltration of inflammatory cells and cytokine production. **a** Fluorescent immunohistochemistry of granulocyte differentiation antigen (Gr)-1 (red) was performed 16 h after cecal ligation puncture (CLP) or sham surgery in A2V (30 mg/kg) or IgG control antibody (IgG)-treated mice (nuclear staining with 4′,6-diamidino-2-phenylindole, blue; autofluorescence is shown in green). Arrowheads indicate Gr-1-positive cells. Representative results for each group are shown ($n = 6–8$). **b** Semi-quantification of whole lung cross-sections by evaluating Gr-1$^+$ cells per high power field (HPF) (HPF = 40× magnification) (** $p < 0.01$). **c** Real-time quantitative polymerase chain reaction from kidneys and lungs (**d**) for interleukin-6 (IL-6) messenger RNA (mRNA) as in the above-described conditions ($n = 8$).

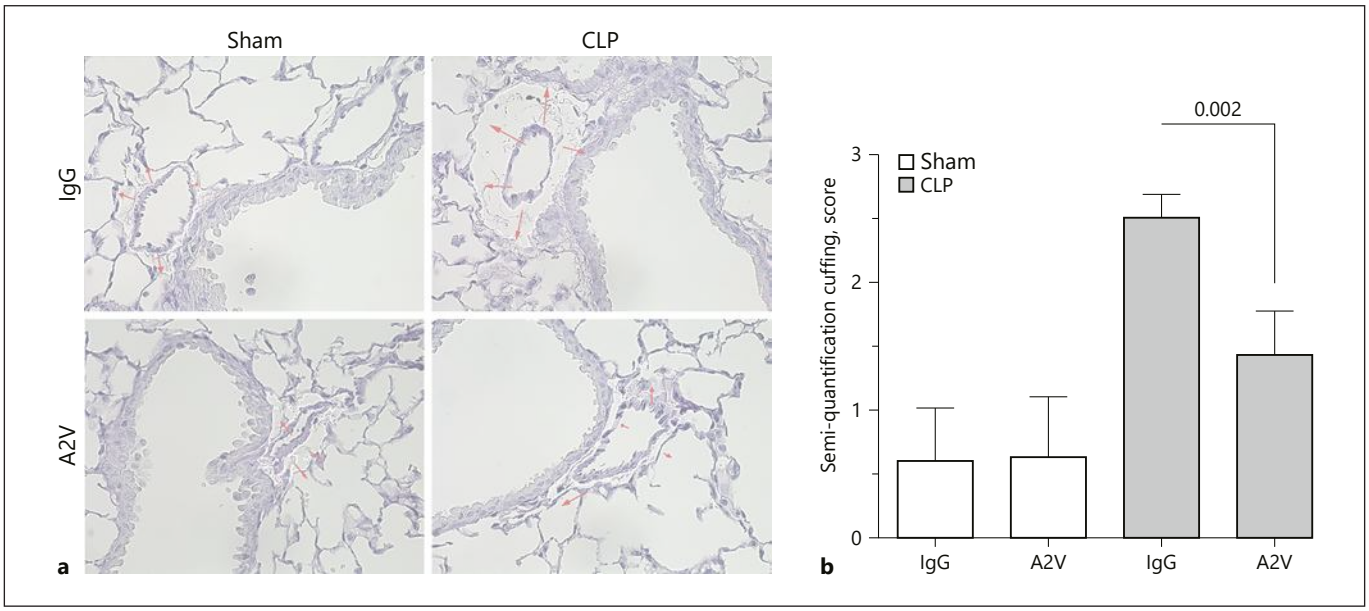

Fig. 3. A2V affects permeability in vivo. **a** Periodic acid-Schiff staining of paraffin-embedded lung tissue 16 h after cecal ligation puncture (CLP) in A2V (30 mg/kg) or IgG control antibody (IgG)-treated mice showing less peribronchial cuffing (red arrows) in CLP mice after A2V treatment compared to control. Representative images of $n = 4–5$/group are shown. All images show bronchus and their corresponding arteriola (as vasa vasorum of the bronchus surrounded by one common adventitia). **b** Semi-quantification of peribronchial cuffing was performed by surveying whole lung sections. Scoring considered the degree of edema and was done by a single blinded operator (0 = no cuffing, 1 = mild cuffing, 2 = moderate cuffing, 3 = severe cuffing).

Table 1. Serum surrogates of organ dysfunction

	Sham		CLP	
	IgG	A2V	IgG	A2V
LDH, U/L	282.0±39.0	290.5±48.6	768.5±124.0 $p = 0.08$*	490.9±76.6
BUN, mmol/L	8.9±0.8	9.5±0.7	17.2±4.6 $p = 0.16$*	9.9±1.3
GPT, U/L	65.2±23.4	52±8.922	158.8±22.8 $p = 0.29$*	127.3±17.1
GOT, U/L	137.7±38.8	126.6±36.6	336.6±35.4 $p = 0.21$*	127.3±17.1

CLP, cecal ligation puncture; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; GPT, glutamate-pyruvate transaminase; GOT, glutamic oxaloacetic transaminase.

* Unpaired *t* test with Welch's correction between the following groups: CLP A2V vs. CLP veh.

For clarity reasons not assigned in table: CLP A2V vs. sham veh.: LDH, $p = 0.03$; BUN, $p = 0.53$; GPT, $p = 0.06$; GOT, $p = 0.01$. CLP A2V vs. sham A2V: LDH, $p = 0.05$; BUN, $p = 0.82$; GPT, $p = 0.003$; GOT, $p = 0.009$. CLP veh. vs. sham veh.: LDH, $p = 0.004$; BUN, $p = 0.10$; GPT, $p = 0.02$; GOT, $p = 0.003$. CLP veh. vs. sham A2V: LDH, $p = 0.004$; BUN, $p = 0.13$; GPT, $p = 0.001$; GOT, $p = 0.002$. Sham A2V vs. sham veh.: LDH, $p = 0.9$; BUN, $p = 0.54$; GPT, $p = 0.62$; GOT, $p = 0.84$.

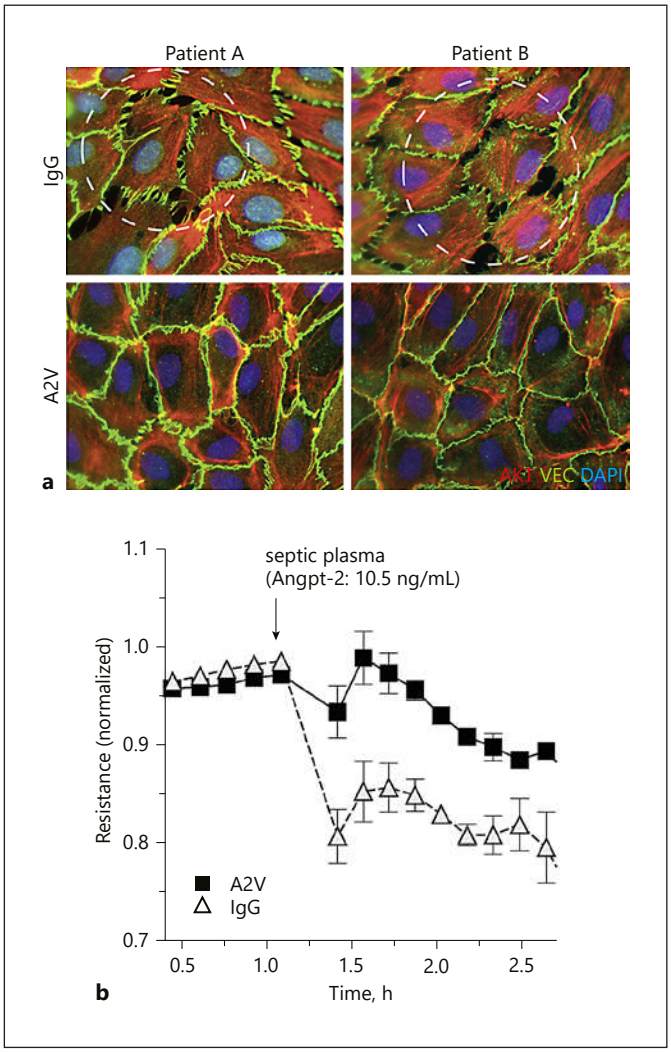

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Fig. 4. A2V affects permeability in vitro. **a** Fluorescence immunocytochemistry staining for vascular endothelial cadherin (VEC, green) and F-actin (red) was performed on confluent human umbilical vein endothelial cells (HUVECs). First, HUVECs were incubated with A2V (30 mg/kg) or control IgG antibody (IgG) and then challenged for 30 min with media supplemented with 5% plasma from two individual septic shock patients. **b** Normalized transendothelial electrical resistance was longitudinally measured in real time in HUVECs with an electric cell-substrate impedance sensing device. HUVECs were first co-incubated with A2V or IgG control antibody followed by stimulation with 5% plasma from septic shock patients (as done for the staining experiment).

What was the rationale to explore yet another angiopoietin/Tie2 modulating strategy given that other approaches have been more or less successfully investigated in recent years (Table 2)? Monoclonal antibodies are nowadays on everyone's lips and represent a drug type used on a daily basis for numerous diseases – in some cases with clinical experience over decades (e.g., CD-20 antibodies against B-cell lymphoma) [23]. More and more novel specific antibodies have been developed and in 2017 alone, a total of 17 novel monoclonal antibodies have been approved by the FDA (around 30 in 2018). Therefore, we believe that this class of drug might have the most realistic potential with regard to drug development from bench to bedside in a translational research context. Yet, exactly this type of drug did not convince with regard to hard end points in preclinical models of sepsis compared to other compounds. For example, Ziegler et al. [16] did investigate a monoclonal Angpt-2 antibody (LC-10, Roche) in murine sepsis. In general, they focused on septic cardiomyopathy and elegantly found improvements in many surrogate end points, but overall survival was only very mildly influenced (10–20%) [16]. In our own hands, blockade of Angpt-2 with a monoclonal antibody did not improve survival in murine sepsis at all. Although, clinically less feasible than a monoclonal antibody approach, most published strategies showed survival benefits around 40–50% (summarized in Table 2) in these preclinical studies. For VEGF blockade, the data were similarly mixed [17, 20–22]. Based on the appealing drug formulation of an antibody and the advanced stage of development of the A2V (four different bispecific antibodies based on the CrossMAb technology are currently in active phase 1/2 clinical trials [18] for colorectal cancer studies and the first is yet to enter phase 3 trial for diabetic macula edema), we aimed at investigating specifically this novel heterodimeric bispecific monoclonal IgG1-cross antibody of Angpt-2 and VEGF.

Indeed, combining both Angpt-2 and VEGF-A blocking strategies in one bispecific antibody, we observed a survival benefit of almost 40%. Again, this effect is in line with other knockout experiments and Angpt/Tie2 or VEGF modulating therapies (Table 2). Although similarly effective, all these potential therapies are at earlier stages of development from bench to bedside, making the A2V antibody approach clinically more attractive. The underlying reason why the combination of Angpt-2 and VEGF-A antibodies might be more effective than individual ones alone remains unanswered by this project. In the oncology field, it has been proposed that tumor microvasculature can develop resistance against single anti-angiogenic agents and that the combination of 2 of them might overcome this problem. If this theory is transferable to an acute setting such as sepsis is unclear.

Given that A2V does not interfere with the infection (here a ruptured colon) per se, yet improves survival by

Table 2. Potential treatment options that interact in the Angpt-2/VEGF axis

Target	Mechanism	Compound	Injury model	Main findings	Reference	Year	Developmental status
Angpt-1	Adenoviral over-expression	AdAng1	LPS/mouse	Improved cardiac function, lung injury, and survival	Witzenbichler et al. [24]	2005	Preclinical
	Angiopoietin-1 variant	COMP-Ang1	LPS/mouse	Reduced vascular leak and inflammation	Hwang et al. [25]	2009	Preclinical
	Angiopoietin-1 variant	COMP-Ang1	LPS/mouse	Decreases acute kidney injury, reduces renal vascular leak and inflammation	Kim et al. [26]	2009	Preclinical
	Agonistic 7-mer peptide	Vasculotide	LPS /mouse	Pretreatment improved survival by 41.4%	David et al. [27]	2011	Preclinical
	Agonistic 7-mer peptide	Vasculotide	CLP/mouse	Survival benefit: pretreatment HR 0.39 (0.19–0.81), rescue treatment HR 0.22 (0.06–0.83)	Kümpers et al. [28]	2011	Preclinical
	Recombinant agonist	rh-Ang1	CLP/mouse	Improvement of MODS and survival time (around 10% vs. 0% after 36 h)	David et al. [29]	2011	Preclinical
	More stable Ang-1 variant	MAT.Ang1	LPS/mouse	Improved vascular leak, microvascular flow, and inflammation	Alfieri et al. [30]	2012	Preclinical
	Agonistic 7-mer peptide	Vasculotide	Pneumococcal pneumonia	Reduction of permeability in lung injury	Gutbier et al. [31]	2017	Preclinical
Angpt-2	Genetic depletion	siRNA	LPS, CLP/mouse	Improvement of kidney function and survival by 50%	Stiehl et al. [32]	2016	Preclinical
	Inhibitor	LC06/LC10	LPS, CLP/mouse	Improves survival by 10–20%, reduced permeability and pericyte loss	Ziegler et al. [16]	2013	Preclinical
	Simvastatin	Inhibition of Angpt-2 biosynthesis	CLP/mouse	Improved survival by 37.5%	Ghosh et al. [33]	2015	Preclinical
	Flunarizin	Inhibition of Angpt-2 biosynthesis and release	LPS/mouse	Improved pulmonary endothelial inflammation and survival by 25%	Retzlaff et al. [34]	2017	Preclinical
	Ang-2/Tie2 clustering → activates Tie2	ABTAA	LPS, CLP, *S. aureus* peritonitis	Improvement of MODS and survival by 50% as pretreatment	Han et al. [35]	2016	Preclinical
Tie2	small molecule inhibitor of VE-PTP – activates Tie2	AKB-9778	LPS/mouse	Stabilizes endothelial junctions	Frye et al. [36]	2015	Preclinical
VEGF	VEGFR1/VEGFR2 antibody	VEGF cytokine Trap (VEGFT)	CLP/mouse	Reduced production of IL-6 and IL-10	Nolan et al. [22]	2004	FDA approval of aflibercept
	Various	Ad-sFlt-1, antibodies against Flk-1 and Flt-1	CLP/mouse	Reduction in VEGF levels, improved leakage, cardiac function, and survival	Yano et al. [20]	2006	Preclinical
	VEGF Trap	sFLT-1 (soluble vascular endothelial growth factor receptor-1)	LPS, CLP/mouse	Attenuated inflammatory responses and improved survival (up to 60 % in pre-treatment)	Tsao et al. [37]	2007	Preclinical
	VEGF-A antibody	Bevacizumab	LPS, CLP/mouse	Improves survival by almost 40% in CLP, decreasing inflammatory responses and endothelial permeability	Jeong et al. [38]	2013	FDA approved

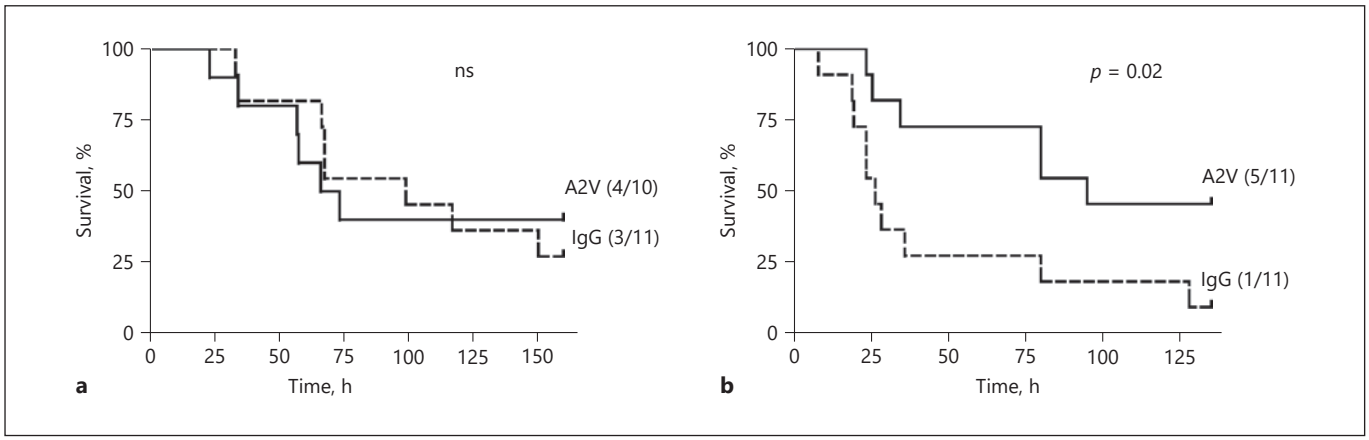

Fig. 5. A2V improves survival in experimental sepsis. Kaplan-Meier survival analysis after CLP-induced sepsis in mice treated with 10 mg/kg body weight A2V (n = 10) or IgG control antibody (IgG) (n = 11) (**a**) and 30 mg/kg A2V (n = 11) or IgG control antibody (IgG) (n = 11) (**b**). Survival was evaluated thrice daily over 5 days.

40%, might sound surprising. We believe that the vasculature that builds a physical border for any inflammatory cell between the blood circulation and sites of (local) infection plays a central role in the pathological (systemic) host response. By blocking excessive endothelial adhesion molecule expression and permeability literally anywhere in the body, A2V might be able to reduce organ failure due to edema and uncontrolled inflammation, thereby improving survival. On the other hand, this means that a complete blockade of these pathways might – at least theoretically – lead to an ultra-strong endothelial barrier unable to guide any inflammatory cell from the circulation to a local infection, a situation that definitely would not be desirable. Therefore, correct dosing and timing is crucial and should be the focus of extensive additional benchwork besides our proof of principle study presented here.

We find the in vitro experiments assessing permeability upon challenge with real septic shock plasma particularly interesting. This plasma must contain countless cytokines and injurious molecules that by themselves might induce endothelial inflammation and permeability. Yet, we observed that dual inhibition of Angpt-2 and VEGF-A alone is sufficient to block the development of vascular leakage. This might indicate that these two pathways indeed play a critical role in septic barrier breakdown.

The study has limitations. First of all, as this was a proof of principle study, we focused on a pretreatment rather than a rescue strategy. The potential negative consequences of blocking vascular inflammation and permeability were just eluded and require further experiments to identify ideal dosing and timing strategies. The synergistic role of Angpt-2 and VEGF-A inhibition was mechanistically not analyzed in our project but deserves further clarification. Methodologically, it is important to mention that the analysis of vascular leakage in vivo by bronchiolar cuffing was only semi-quantitative.

In conclusion, this study is the first that investigates a novel heterodimeric bispecific monoclonal IgG1-cross antibody of Angpt-2 and VEGF-A in experimental murine sepsis. It shows improved endothelial inflammation and permeability, ultimately leading to a survival benefit of almost 40%.

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Statement of Ethics

This study complies with the guidelines for human studies. All research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Disclosure Statement

The authors declare that they have no competing interests directly related to this study. S.D. has received honoraria from DSO, Cytosorbents, Orion, and CSL Behring.

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Author Contributions

J.H., C.S., K.T., and J.R. performed experiments that were designed by S.D. J.H., C.S., and S.D. analyzed and interpreted all data and wrote the manuscript. C.v.K. provided human umbilical veins for endothelial cell isolation and proofread the manuscript. H.H. helped troubleshooting and interpreting data and proofread the manuscript.

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